Original Article

Life Science

Effectiveness of Ozone in Inactivating *Listeria monocytogenes* from Milk Samples

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ABSTRACT [ENGLISH/ANGLAIS]

Inactivation of *Listeria monocytogenes* using ozonation was studied in raw milk and various branded milk samples in and around Coimbatore city. Total of 20 milk samples were obtained from super markets and other places. The PALCAM agar was used in the study to enumerate *L. monocytogenes* from raw milk and various branded milk samples. Results indicate that all the samples are positive prior to the ozonation process. A controlled flow rate 0.5 m/l of oxygen was used to produce 0.2g/h of ozone. The milk samples were ozonated at 0, 5, 10, and 15 minutes. After treatment the samples are inoculated and *L. monocytogenes* were enumerated by using listeria PALCAM agar. After 15 minutes ozonation *L. monocytogenes* were completely eliminated from milk samples. Before and after ozonation the samples were analyzed for protein, carbohydrate, and calcium content. After treatment the nutritional values were slightly different in the milk samples.

Keywords: *Listeria monocytogenes*, Ozonation, Pal cam Agar, Pathogenic microbes

RÉSUMÉ [FRANÇAIS/FRENCH]

L'inactivation de Listeria monocytogenes en utilisant l'ozonation a été étudiée dans le lait cru et de divers échantillons de lait de marque dans et autour de Coimbatore ville.Total de 20 échantillons de lait ont été obtenus à partir de super marchés et autres lieux. L'agar PALCAM a été utilisée dans l'étude d'énumérer L. monocytogenes dansle lait cru et de divers échantillons de lait de marque. Les résultats indiquent que tous les échantillons sont positifs avant le processus d'ozonation. Un débit contrôlé de 0,5 m / l d'oxygène a été utilisé pour produire 0,2 g / h de la couche d'ozone. Les échantillons de lait ont été ozonée à 0, 5, 10 et 15 minutes. Après traitement, leséchantillons sont inoculés et L. monocytogenes ont été dénombrés par l'aide de gélose PALCAM Listeria. Après 15 minutes d'ozonation L. monocytogenes ont été complètement éliminés à partir d'échantillons de lait. Avant et après ozonation les échantillons ont été analysés pour les protéines, des glucides et la teneur en calcium.Après traitement, les valeurs nutritionnelles étaient légèrement différents dans les échantillons de lait.

Mots-clés: *Listeria monocytogenes*, l'ozonation, Pal cam Agar, les microbes pathogens

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INTRODUCTION

Listeria are small, Gram positive, nonsporulating, facultatively, anaerobic rod, which measures 1-2 x 0.5 microns and shows characteristic tumbling motility at 25° C. Listeria are able to multiply high salt concentrations (10 % NaCl) and broad range of pH (pH 4.5 – 9) and temperature (0 -45°C, optimum 30 to 37°C). The genus Listeria includes six species namely *L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seeligeri, L. grayi.* Of these *L. monocytogenes* is an opportunistic pathogen in humans and animals [1]. *L. monocytogenes* is the causative agent of listeriosis. It is one of the most virulent foodborne pathogens, with 20 to 30 percent of clinical infections resulting in death [2]. Responsible for approximately 2,500 illnesses and 500 deaths in the United States (U.S.) annually, listeriosis is the leading cause of death among foodborne bacterial pathogens, with fatality rates exceeding even that of Salmonella and Clostridium botulinum [3].

Many methods have been used to degrade the *L. monocytogenes* such as Pasteurization, High pressure processing, UV, and Ozonation methods. These methods are effective in degrading the *L. monocytogenes* from milk samples. The purpose of this investigation is to isolate *L. monocytogenes* from milk samples and destroy it by using ozonation process without any changes in the nature of raw milk samples.

MATERIALS AND METHODS

Sample collection

Twenty milk samples were obtained from super markets and various places in and around Coimbatore city during the period of December 2008-May 2009, the milk samples were collected in two months once Table 1 shows, the milk samples contain *L. monocytogenes.* Samples were brought under cold conditions in ice-boxes from the place of collection to the laboratory and analyses are carried out immediately.

TABLE 1

Table 1 shows the *Listeria* positive sample sites for raw and branded milk

SN	Sample site	Visit Month		
		December	February	May
1	Raw milk 1	LM	LM	LM
2	Raw milk 2	LM	LM	LM
3	Raw milk 3	LM	LM	LM
4	Raw milk 4	LM	LM	LM
5	Raw milk 5	LM	LM	LM
6	Raw milk 6	LM	LM	LM
7	Raw milk 7	LM	LM	LM
8	Raw milk 8	LM	LM	LM
9	Raw milk 9	LM	LM	LM
10	Raw milk 10	LM	LM	LM
11	Raw milk 11	LM	LM	LM
12	Raw milk 12	LM	LM	LM
13	Raw milk 13	LM	LM	LM
14	Raw milk 14	LM	LM	LM
15	Raw milk 15	LM	LM	LM
16	Branded milk A	LM	-	LM
17	Branded milk B	LM	LM	LM
18	Branded milk C	LM	LM	LM
19	Branded milk D	-	LM	LM
20	Branded milk E	-	LM	LM

LM = *Listeria monocytogenes;* SN = *Serial number*

Ozone Treatment and Experimental Set-up

The experimental set up was consists of an oxygen concentrator, ozone generator. Controlled flow rate of 0.5 l/min of oxygen were used to produce 0.2g/h of ozone. An ozonation chamber with 100 ml column was used in the study. Teflon tube was used for connecting the ozone outlet port from the ozone generator to the ozone reaction chamber. The 49 ml of milk samples has been taken and 1 ml of *L. monocytogenes* culture has been added in the column. Samples were ozonated at 0, 5, 10 and 15 minutes after the commencement of ozonation the samples were inoculated and *L. monocytogenes* were enumerated by plating in Listeria identification agar and incubated at 35 °C for 24 hours. After incubation five typical colonies from these media were transferred to LB broth and incubated for 24 hours at 30 ◦ C. The LB broth culture was determined by using optical density at 600 nm [4].

Isolation and identification of *L. monocytogenes*

The Food and Drug Administration (FDA) method were used for the detection of *L. monocytogenes.Listeria* Enrichment Broth (LEB) of 225 ml without selective supplement (PALCAM) was added to 25 ml milk sample, and incubated at 30°C for 4 h. Then, Listeria Enrichment Broth Selective Supplement was added and incubated for another 44 h, for a total of 48 h incubated at 30 °C. After 24 and 48 h of incubation the test culture was streaked onto PALCAM agar plate and it was incubated for 24– 48 h at 35 °C. After incubation, five typical colonies from these media were transferred to LB broth and incubated for 24–48 h at 30 °C. The purified isolates were identified by classic tests such as, Gram staining, examination of catalase activity, rotating or tumbling motility, oxidase test, MR-VP test, indole production test, urease test, citrate utilization test, and haemolysis zone on blood agar, carbohydrate fermentation tests in Purple Carbohydrate Broth, and CAMP test [5].

Nutritional Analysis

All the milk samples were estimated for nutritional values such as protein, carbohydrate, and calcium before and after ozonation. The estimation procedures by Lowry's method for protein, Anthrone method for carbohydrate, and Flame photometer method for calcium were followed.

RESULTS AND DISCUSSIONS

Isolation and Identification of *L.monocytogenes*

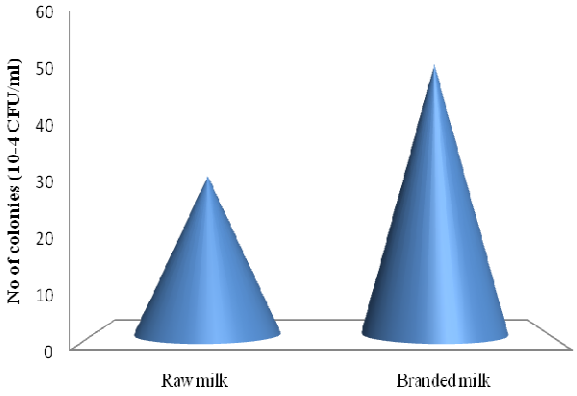
The isolation of *L. monocytogenes* from raw milk samples and branded milk samples are shown in Figure 1 respectively. In raw milk samples 31% colonies were isolated. In branded milk samples 53% colonies were isolated. All the isolates were analyzed by standard biochemical test, such as Gram positive, catalase positive, Tumbling motility, oxidase negative, carbohydrate fermentation, indole negative, MR positive-VP negative, citrate negative, urease negative, and CAMP positive. Results indicate that both raw and branded milk samples were contaminated by *L. monocyotgenes and* the incidence of *Listeria* in branded milk was higher than that of raw milk samples, possibly because raw milk is a larger

processing plant and receives raw milk from a number of different farms. Whereas branded milk also a larger processing plant and some time receives branded milk from post pasteurized. This higher rate of contamination may be explained by the fact that the branded milk samples here came from a post pasteurized or improper pasteurization [6].

In this study *Listeria spp*. was detected in pasteurized milk and raw milk, demonstrating the potential for contamination of pasteurized milk with potentially pathogenic *L. monocytogenes*. Although other *Listeria sp*, such as *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, and *L. greyi*, are non-pathogenic. *L .monocytogenes*, are believed to co-exist with other *Listeria sp* in the same environmental niches, so the isolation of any *listeria spp* should be seriously viewed. According to the FDA dairy safety initiative programme, 2.7% of milk processing plants were found to have end products that were contaminated with *Listeria sp* [7].

FIGURE 1

Figure 1 shows the isolation of *L. monocytogenes* from raw milk samples and branded milk samples



Degradation of *Listeria* from milk samples

Results indicate that *L. monocytogenes* was present in raw milk and pasteurized milk samples. Ozonation process was used to eliminate the *L. monocytogenes* from milk samples. The mean bacterial concentration for *L. monocytogenes* after 5, 10 and 15 minutes of ozonation are illustrated in Table 2 and 3 for raw and branded milk samples respectively. The degradation of raw milk samples ozonated at 5 minutes ranged from 0.5995 to 0.8967, ozonated at 10 minutes ranged from 0.2528 to 0.0898 and ozonated at 15 minutes the *L. monocytogenes* were completely eliminated. The branded milk samples ozonated at 5 minutes ranged from 0.291 to 0.762 and ozonated at 10 minutes to15 minutes the *L. monocytogenes* were entirely eradicated and it reveals that the *L. monocytogenes* were completely degraded within 15 minutes of ozonation. Hence, the ozonation method is very effective for inactivation of *L. monocytogenes*.

TABLE 2

Table 2 shows the degradation of *L. monocytogenes* in raw milk samples using ozonation

Sample	Before Ozonation (OD at 600 nm)	After ozonation (OD at 600 nm)		
		5 min	10 min	15 min
Raw milk 1	1.105	0.689	0.23	0
Raw milk 2	1.1054	0.5995	0.1034	0
Raw milk 3	1.1054	0.6541	0.104	0
Raw milk 4	1.1054	0.755	0.1013	0
Raw milk 5	1.1054	0.7765	0.1718	0
Raw milk 6	1.1054	0.7494	0.1714	0
Raw milk 7	1.1054	0.8265	0.1272	0
Raw milk 8	1.1054	0.7972	0.0898	0
Raw milk 9	1.1054	0.8347	0.1732	0
Raw milk 10	1.1054	0.8967	0.0939	0
Raw milk 11	1.1054	0.7308	0.2183	0
Raw milk 12	1.1054	0.6924	0.2528	0
Raw milk 13	1.1054	0.845	0.1682	0
Raw milk 14	1.1054	0.812	0.1064	0
Raw milk 15	1.1054	0.874	0.2355	0

TABLE 3

Table 3 shows the degradation of *L. monocytogenes* in branded milk samples using ozone

Sample	Before Ozonation (OD at 600 nm)	After ozonation (OD at 600 nm)		
		5 min	10 min	15 min
Branded Milk A	1.105	0.514	0	0
Branded Milk B	1.105	0.762	0	0
Branded Milk C	1.105	0.291	0	0
Branded Milk D	1.105	0.379	0	0
Branded Milk E	1.105	0.582	0	0

Nutritional Values in Milk Samples before and after Ozonation

The raw and branded milk samples were estimated for nutritional values such as protein, carbohydrate, and

calcium before and after ozonation to determine whether ozonation affects the nutritional values of milk. All the milk samples were ozonated at 5, 10, and 15 minutes. Protein values for the raw milk samples were ranged from 98 mg/100 ml to 256 mg/100 ml and the branded milk samples were between 98 mg/100 ml and 143 mg/100 mL. Carbohydrates values for the raw milk samples were ranged from 2.0 mg/100 ml to 3.7 mg/100 ml and branded milk samples were between 1.6 mg/100 ml, and 7.8 mg/100 ml. Calcium values for the raw milk samples were ranged from 150 mg/100 ml to 190 mg/100 ml and the branded milk samples were of 120 mg/100 ml to 270 mg/100 ml.

FIGURE 2

Figure 2 shows the effect of ozone on protein from raw milk samples

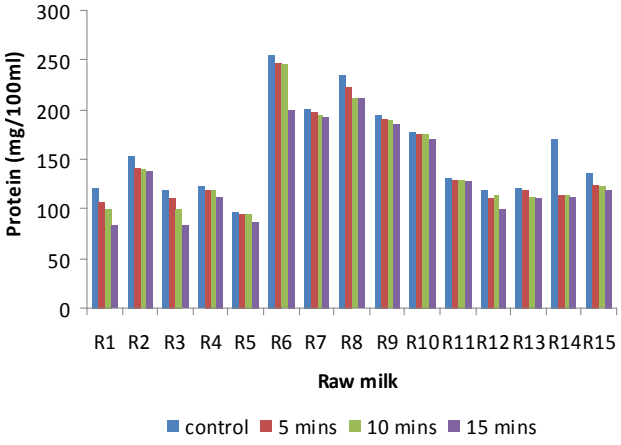


FIGURE 3

Figure 3 shows the effect of ozone on protein from branded milk samples

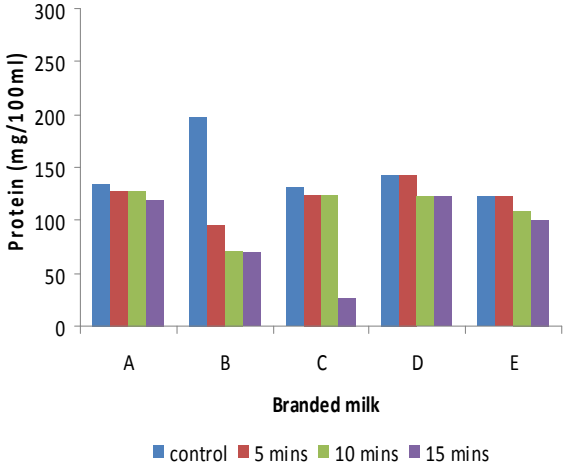


FIGURE 4

Figure 4 shows the effect of ozone on carbohydrates from raw milk samples

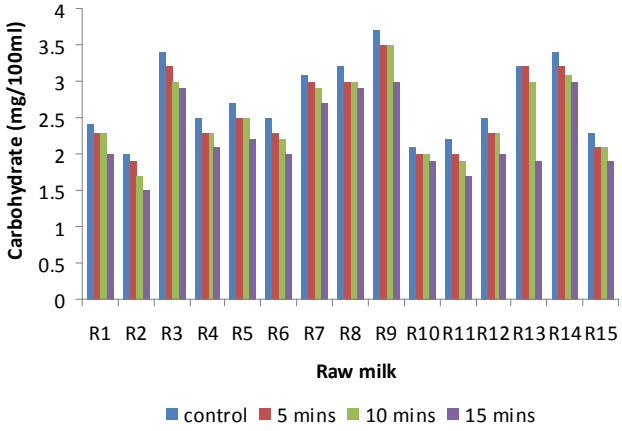
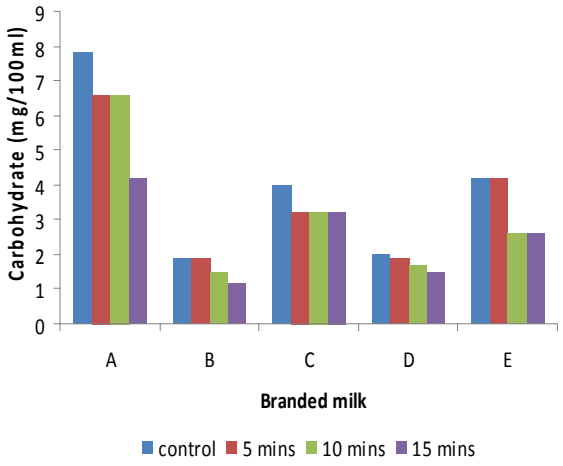


FIGURE 5

Figure 5 shows the effect of ozone on carbohydrates from branded milk samples



Protein values were plotted in figure 2 and 3 and it was found that after 15 minutes of ozonation around 15 % reductions in protein. Carbohydrate values also reduced around 15 % in raw samples after 15 minutes of ozonation; it was shown in figures 4 and 5. The comparative differences in the calcium values after ozonation were only 10%. and the results were plotted in the figures 6 and 7. The reason for tiny reduction in nutritional values such as protein, carbohydrate, etc., may be excess of time consumption during ozone treatment causes undesirable protein denaturation, non-enzymatic browning, and loss of vitamins and volatile flavour compounds [8].

FIGURE 6

Figure 6 shows the effect of ozone on calcium from raw milk samples

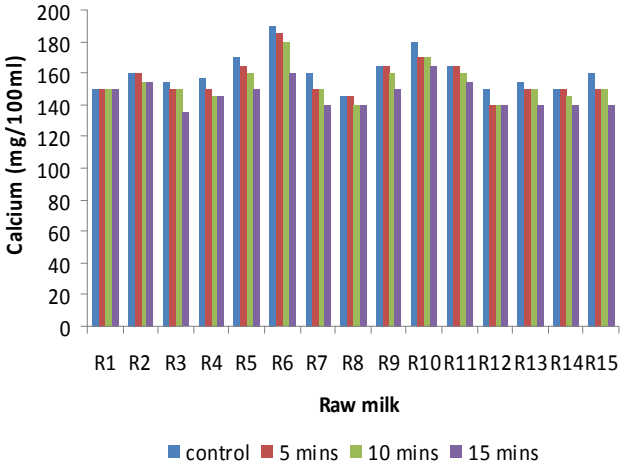
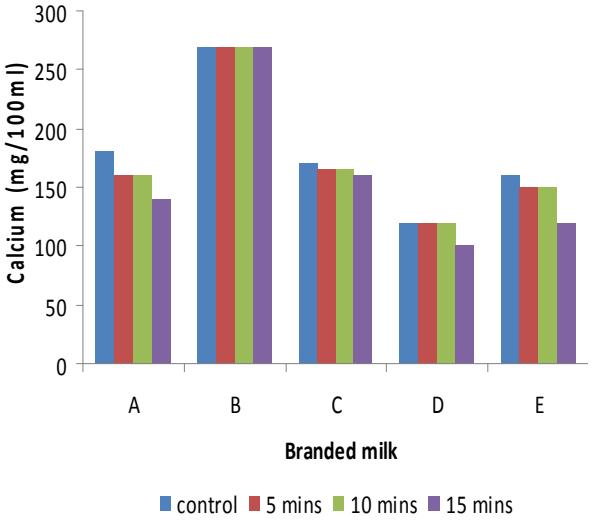


FIGURE 7

Figure 7 shows the effect of ozone on calcium from branded milk samples



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Nil

CONFLICT OF INTEREST

No conflict of interest was declared by authors.